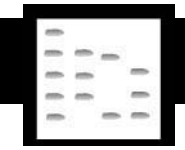
CHROMATOGRAPHY



Automation

E. Vérette, Gilson, Villiers-le-Bel, France

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The last 20 years have seen an important increase in published papers on chromatography, reporting fully automated systems or complete online techniques. This shows a strong interest in automation in chromatography to obtain faster, safer, more cost-effective and convenient analytical procedures, that satisfy regulatory compliance and good laboratory practice directives. Automation in chromatography takes place in the more general laboratory automation context, which is the application of computing, robotics, electronics and mechanical engineering technologies to laboratory problems for enhanced throughput, reproducibility and traceability. This has been made possible as a result of considerable improvements in equipment and techniques, from autosamplers, sample processors and reliable eluent delivery devices, to universal or more specific detectors, collectors, single point control software able to collect and handle accurate data (**Figure 1**) and, last but not least, laboratory information management systems.

The chromatographic techniques whose automation is principally discussed in this article are gas chromatography (GC), high performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC). Other chromatographic methods such as planar chromatography can also be automated, but full automation is more difficult to achieve.

One of the most important aspects of automation is the introduction of interfaces between instruments and computers, and centralized user-friendly software for system control and data handling. The main qualities required for interfaces, designed to collect analog signals and digitize them to reflect detector output accurately, are good sampling rates and high resolution. Other important aspects of automation are directly related to the evolution of individual instruments, and the possibility of coupling them online. This article is not an exhaustive description of automation in chromatography, but highlights the principal trends in this field. It emphasizes sample preparation and application in chromatographic systems, multidimensionality and fully automated solutions widely used today such as high throughput screening and preparative chromatography.

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Autosamplers

Not far behind computerized data acquisition and analysis, autosamplers are one of the biggest factors in automation, allowing long unattended series of analyses, therefore releasing the operator for other tasks. Autosamplers also give considerable improvement in reproducibility. Autoinjectors for HPLC, GC

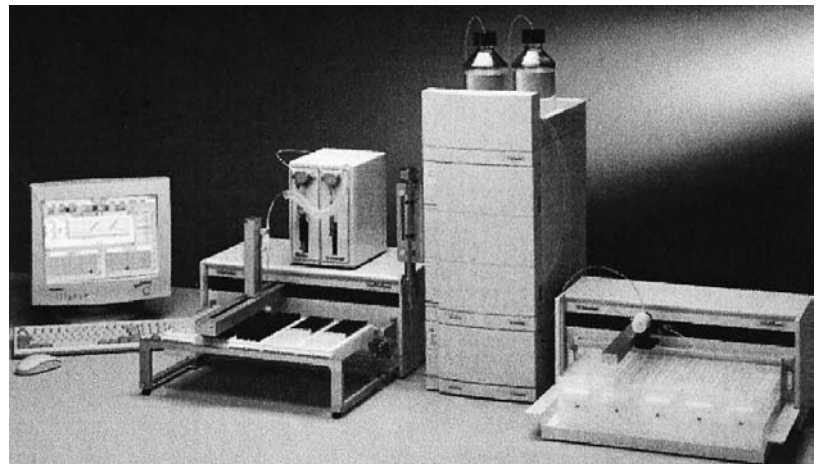

Figure 1 A fully automated HPLC system including an autoinjector, a pump, a detector and a collector controlled by centralized software.

and SFC are generally of Cartesian displacement or of carousel types.

High Performance Liquid Chromatography

HPLC is considered to be the major chromatographic technique available today for involatile or heat-sensitive substances. Sample injection is commonly performed using a switching valve via an external or internal sampling loop. Sample containers range from simple vials with a wide range of volumes to different types of microtitre plates (96 and 384 wells), with the option of temperature control. Using 384-well microtitre plates, some modern instruments can store and handle over 4600 samples unattended. Many autosamplers have three injection modes, total, partial and centred loop filling, depending on the sample volume limitations. In recent years the need for faster analysis has been a major consideration; therefore, the shortest time required between two injections (injector cycle time) appears to be an important criterion for autoinjectors, and may be less than 20 s in the fastest. Other important criteria are injection precision and the level of carryover, with typical values below 0.3% (relative standard deviation) and 0.02% respectively for the best instruments.

Gas Chromatography

Because of its extremely high separation efficiency, speed of analysis and wide range of sensitive detectors, GC and particularly capillary GC is an appropriate procedure for analysing many compounds in a great variety of applications, provided these compounds are volatile, thermally stable and with reasonable polarities to avoid problems encountered with derivatization procedures. However, until recently its major critical point was the injection of large volumes, especially when traces of organic compounds have to be analysed, and the suitability of the sample solvent. The injector influences accuracy, precision, resolution and analyte recovery. The use of new automatic injection devices has provided the means to enhance the limit of detection significantly. Five modes of operation (split, split-less, direct, cold on-column injection, and programmed temperature vaporizer) allow maximum injector flexibility. Among these, the programmed temperature vaporizer (PTV) is of major utility for the automated injection of large sample volumes (**Figure 2**). This technique is based on the selective elimination of the solvent, while simultaneously trapping the components with a much lower volatility. Liquid samples are introduced into a large capacity, sorbent-packed liner inserted in the injector head. By temperature-programming the liner, the solvent is vented through the split exit while analytes are retained. The split valve is then closed and the injector is rapidly heated to transfer the analytes to the GC column. With such a technique, injection volumes in split-less capillary GC can be increased up to 1 mL and this procedure therefore brings enormous improvements in limits of detection.

Static or dynamic headspace sampling, is another interesting way of injecting volatile compounds from complex matrices into a GC. In this process, the vapour in equilibrium above a sample held at constant temperature in a sealed vial is drawn and analysed. Headspace GC is used to analyse a wide range of compounds in solid and liquid complex matrices, and fully automated devices are available today with computer-activated heating and pneumatic systems.

Sample Preparation

For a long time, sample preparation has been considered to be the bottleneck of the analysis. However, the advances of automation in this area have until recently been distinctly more modest compared with other areas. The move towards automation in sample preparation occurred for several reasons, including higher productivity, fewer and more skilled laboratory personnel, better analytical results and less hazardous conditions. Basically, there are two almost complementary types of equipment, anthropomorphic arms and Cartesian liquid handlers, used according to the nature and quantity of samples to be processed and the preparation complexity (**Figure 3**). Anthropomorphic arms are capable of grabbing objects and moving them from one place to another with high accuracy. They need a set of dedicated stations to carry out different operations such as grinding, homogenizing, drying, weighing, etc. The principal preparation steps used with Cartesian (XYZ) samplers are dilution, addition of reagents or internal standards and mixing. With additional equipment they can also perform temperature control, evaporation or filtration.

Solid-phase Extraction (SPE)

SPE has become a technique of choice for sample clean-up and trace enrichment in the fields of pharmaceutical, clinical, food and environmental analysis. Indeed, it offers better versatility and selectivity than other sample preparation techniques. Compared with liquid–liquid extraction, it is quicker, it requires much less solvent, it prevents possible formation of emulsions, sample volumes can be smaller, and it is

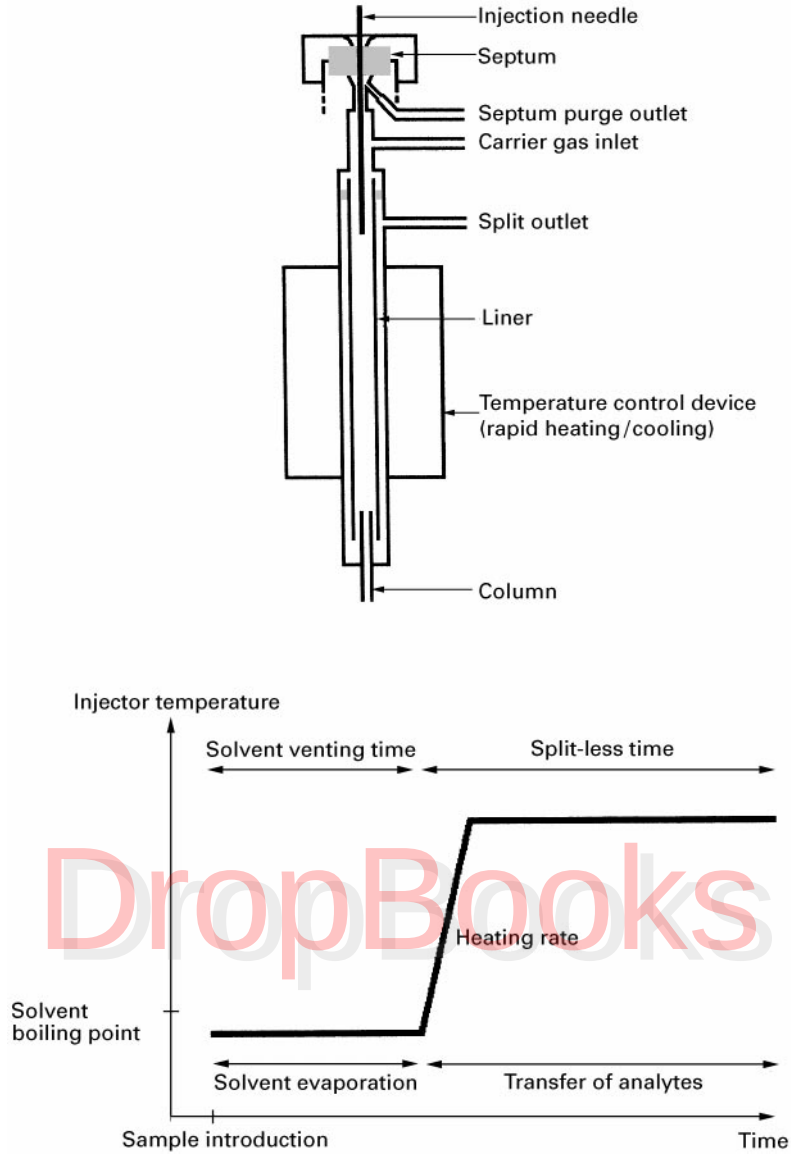

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Figure 2 Schematic representation of a PTV injector and temperature profile of injection.

much more amenable to automation. The US Environmental Protection Agency has adopted more than 20 SPE methods as alternatives to liquid–liquid extraction. SPE formats range from traditional disposable extraction columns and membrane discs to 96-well plates and SPE pipette tip configurations. Today, a wide range of sorbents is available to increase selectivity and/or simplify the SPE procedure, from silica- to polymer-based phases, including speciality phases with mixed-mode functionality.

An experimental SPE procedure typically follows four steps – conditioning, loading, washing and eluting. With offline systems using disposable columns, liquids go through the extraction columns either by aspiration (vacuum manifolds), or by positive air pressure; the latter can be completely automated. **Figure 4** shows typical SPE automation, based on the mobility of a dedicated rack consisting of three parts. After elution and a final mixing step, the eluate can be automatically introduced into the chromatograph via an injection valve for analysis.

Fully automated offline SPE brings an easy transfer of manual methods, which results in a quick transposition of hundreds of existing protocols with little additional development. Since the introduction of the large volume GC injection with a PTV injector interface, automated SPE has been extended to numerous GC applications. Some SPE instruments

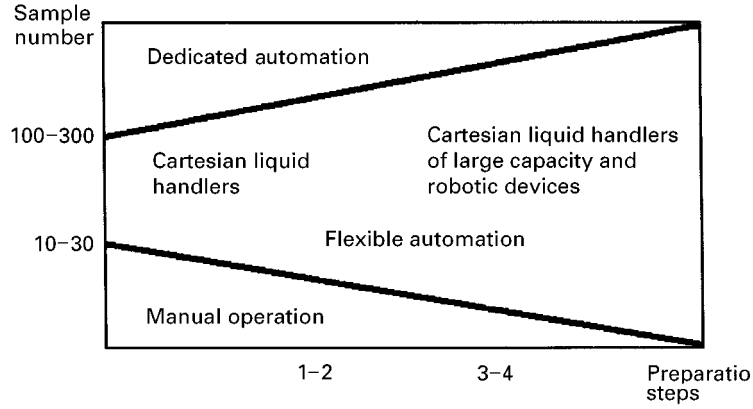

Figure 3 Diagram of estimated automation requirements according to sample number and preparation complexity.

propose a flexible process to develop new methods such as the possibility for multicollection, multi-modal SPE or column drying, and more recently the possibility of using 96-well SPE microplates with a multiple probe equipment.

In online configurations, an extraction cartridge is inserted as part of the chromatographic equipment, and is directly connected to the high pressure stream of the mobile phase. Such automated SPE equipment can use either the same cartridge for multiple extractions, or carry out automatic cartridge exchange. Online SPE processes are also known for pre-column concentration techniques, and may involve column switching or coupled-column procedures. The online approach provides high throughput and preserves the sample integrity, but the technique is still dependent on particular applications and the reproducibility of packing materials.

Solid-phase Microextraction (SPME)

SPME, introduced by Pawliszyn in the early 1990s, is a sample preparation and introduction method in which analytes migrate from a liquid, headspace or air sample on to a polymer (principally polydimethylsiloxane or polyacrylate) that is coated on a fused silica rod. The fibre is then displaced into a thermal or solvent desorption interface for analysis respectively by GC or HPLC. All these steps can be fully automated using autosamplers specifically adapted to SPME, such as the recent in-tube SPME device for HPLC. This technique, which is used repeatedly, provides fast, easy-to-use and inexpensive sampling, and eliminates organic solvents. Initially applied to the analysis of relatively volatile environmental pollutants in simple matrices, it now allows sampling of a broader range of analytes, including drugs in more complex media, with improved analytical performance.

Valve Switching Devices for Sample Preparation

Valve switching devices allow variable combinations of columns, mobile phases and/or detectors, which contribute to the overall system selectivity. When large numbers of substances are present in complex samples like biological fluids, and the compounds of interest are at very low concentrations, such devices are used sequentially to carry out sample clean-up and trace enrichment. Different set-ups are available depending on the type of application, permitting the transformation of offline multi-step methods to single-step procedures. The simplest configuration uses one pre-column between the sample injector and a six-port valve which rotates after venting the unwanted components to waste (straight-flush mode). A more powerful process can be achieved by operating in backflush mode, but necessitates additional equipment. In recent years, switching devices have regained interest due to the introduction of the bimodal restricted access sorbents. Designed for the deproteinization of biological samples, restricted access sorbents are characterized by a hydrophilic external surface incorporating reversed-phase coated pores. Interfering proteins are excluded from the pores by hydrophilic and size exclusion interactions, and low molecular analytes like drugs and metabolites are extracted and enriched on the bonded phase in the sorbent pores. This new type of separation medium is best used in the form of an extraction cartridge fitted to the six-port valve of a sampling injector, before analysis with a more conventional column.

A schematic diagram of a fully automated set-up including an auto-injector with two switching valves is shown on **Figure 5**, where a preliminary online filtration step is performed to extend the lifetime of the cartridge when analysing crude samples. After

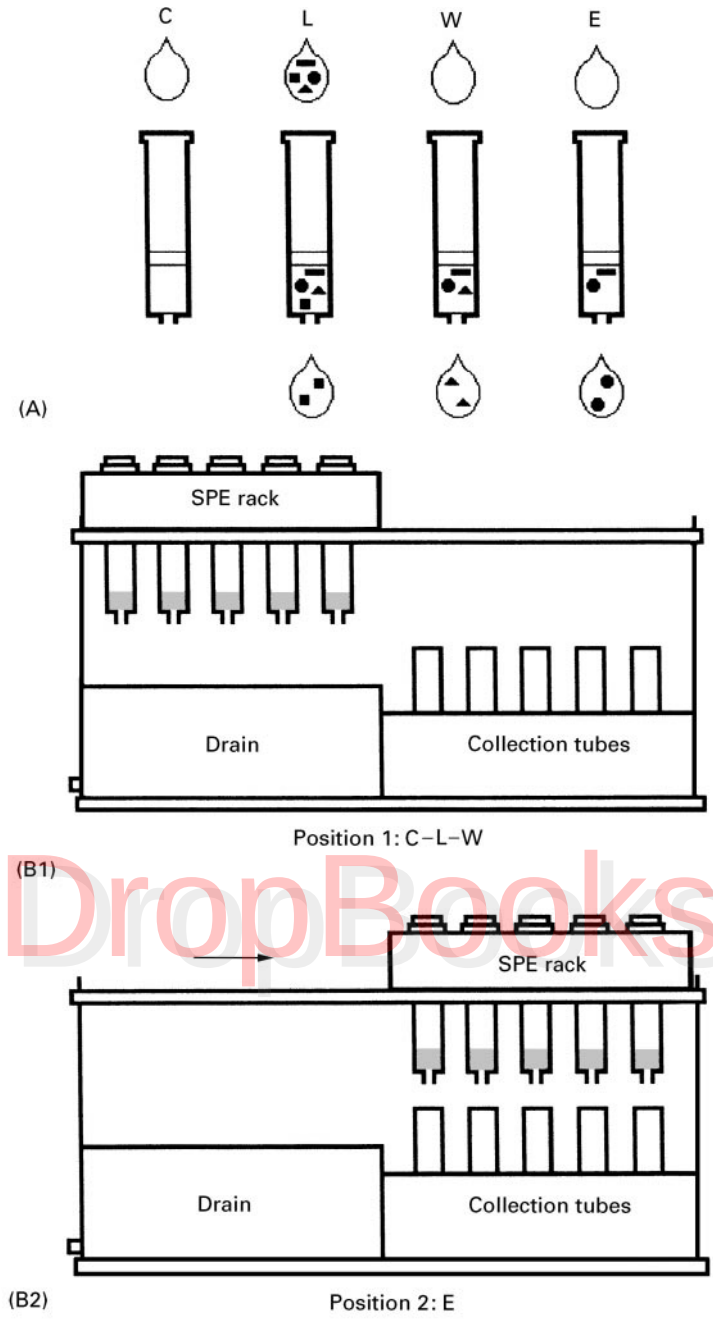

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Figure 4 Automated solid-phase extraction based on ASPEC™ (Gilson) technology. (A) The four basic steps: conditioning (C), loading (L), washing (W) and eluting (E). (B1) Conditioning, loading and washing over the drain container. (B2) Eluting into the collection tubes.

injection the filtration device is regenerated with suitable solvents in backflush mode. Membrane-based sample clean-up such as dialysis, electrodialysis and ultrafiltration is another efficient and innovative way of discarding macromolecular and microparticulate matters before analysis. These devices can be automated and associated online with a chromatographic system, mostly HPLC, using switching valves.

Figure 6 shows a commercially available system automatically performing liquid transfer steps like addition of internal standards or derivatization, dialysis and trace enrichment from crude samples, for the online routine analysis of a large number of samples. This instrument consists of a XYZ autosampler, equipped with a dual-syringe pump, a flat-bed dialyser inserting a porous membrane and two six-port valves. After each analysis, the system is completely regenerated.

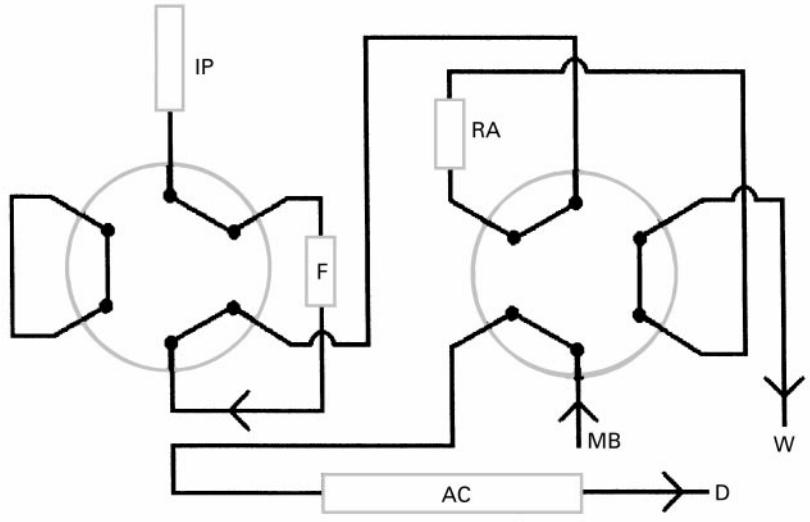

Figure 5 Online filtration and clean-up of biological fluid samples using valve-switching devices. AC, Analytical column; D, detector; F, filtration cartridge; IP, injection port; MB, mobile phase; RA, restricted-access sorbent cartridge; W, waste.

Supercritical Fluid Extraction (SFE)

Supercritical fluids have physical and chemical properties that make them particularly useful for extraction of analytes from solid and liquid matrices. Above their critical temperature, they can be compressed to increase their density and therefore their solvating power; moreover, they keep gas-like viscosity and diffusivity, and they can easily be evaporated by lowering the pressure. Because of these properties, SFE has received much attention as an alternative to Soxhlet extraction. Automated SFE can be incorporated online with various analytical techniques such as HPLC, GC or SFC.

Multidimensionality

In the analysis of complex samples, a single analytical column combined with a selective detector does not always provide the required sensitivity and selectivity. Automated multidimensional chromatography has emerged as a convenient answer to these

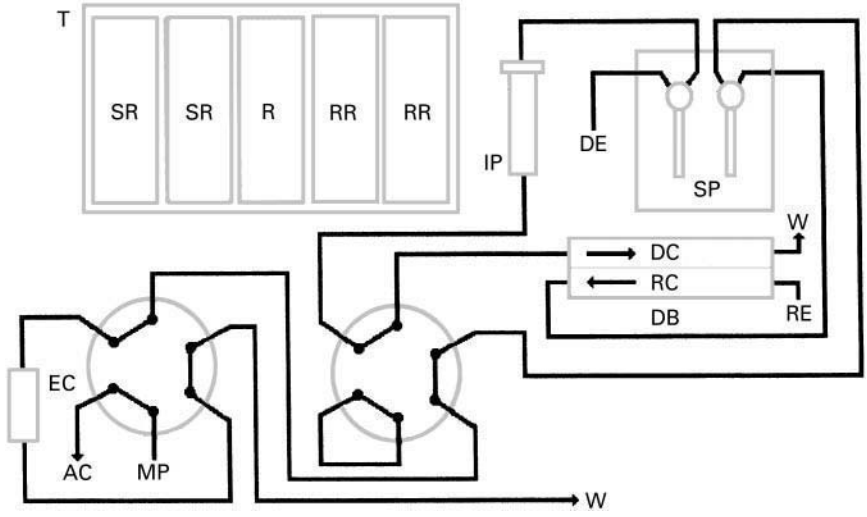

Figure 6 Online dialysis and trace enrichment of liquid samples using ASTED™ (Gilson) technology. AC, Analytical column, DB, dialyser block; DC, donor channel; DE, dilutor eluent; EC, enrichment cartridge; IP, injection port; MP, mobile phase; R, reagent rack; RC, recipient channel; RE, recipient eluent; RR, result rack; SP, dual-syringe pump; SR, sample rack; T, tray; W, waste.

problems. Multidimensional separation techniques constitute a powerful class of methods in which two or more independent steps are linked together. Roughly, multidimensionality includes two major branches: hyphenation and coupled-column techniques. A hyphenated instrument can be described as the combination of two (or more) instruments automated as a single integrated unit via a hardware interface whose function is to reconcile the output limitations of one instrument and the input limitations of the other.

Couplings of Chromatographs and Spectral Technique Devices

In recent years, numerous publications have described coupled techniques associating chromatographic instruments like HPLC, GC or SFC with sophisticated detectors or spectrometric devices, such as mass spectrometers (MS), MS coupled with diode array detector (DAD), MS-MS, Fourier transform infrared and nuclear magnetic resonance. However, for these online couplings, complete automation is generally required, and they are only feasible if suitable interfaces are available. Among these combinations, GC-MS and HPLC-MS are the most widely used in laboratories.

Gas Chromatography–Mass Spectrometry MS data provide the qualitative information for identification and characterization of sample components that is lacking in most other GC detectors. The availability of highly inert, durable and selective fused-silica columns and the ease of coupling GC systems with relatively low cost mass spectrometers make GC-MS a method of choice for numerous analyses, and this automated combination is now a routine analytical technique in many laboratories. The main restriction is that the analytes must be sufficiently volatile and thermostable for transfer to the vapour phase without decomposition, or that they can be derivatized to provide these properties.

High performance liquid chromatography–mass spectrometry Of all LC detectors, MS is a very powerful confirmatory tool for screening applications, and is widely used for impurity identification or quantification of drugs and metabolites in biological fluids. Nevertheless, no method of interfacing HPLC and MS exists that can be universally applied to all types of analyses. However, considerable progress has been made recently in the development of soft ionization techniques such as matrix-assisted laser desorption ionization, and also in sample introduction devices like particle beam, electrospray/ion spray or atmospheric pressure chemical ionization interfaces. When nonvolatile buffers like phosphates or ion-pairing agents are used, removal of these additives can be automatically performed, changing the phase system with additional equipment, using coupled-column and valve-switching techniques.

Couplings of Chromatographic Systems

These techniques are versatile and powerful, and are well suited to the separation of multicomponent mixtures. They include zone-cutting methods, in which fractions from one chromatographic column are usually transferred via a loop installed on a switching valve, to one (or more) secondary column(s) in series for additional separation(s), or to another chromatographic system. In LC-LC, a special area of interest of column-switching is the resolution of enantiomeric drugs in biological fluids, by coupling chiral systems to conventional reversed-phase columns. Other useful associations combine size exclusion or ion chromatography with reversed-phase chromatography. Parallel settings using switching valves are also possible for column and mobile-phase selection. Such systems have been reported for fully automated, rapid and easy method development to analyse different types of compounds, including enantiomers.

LC-GC is a very convenient combination which associates the selectivity of LC with the high efficiency capillary GC. Moreover, GC possesses many sensitive and specific detectors. Such a coupled technique enables the analysis of samples with complex matrices, and considerably prolongs the GC column lifetime. The first set-up was described by Majors in 1980; since then important improvements in development have been achieved. With this transfer technique, specific attention must be paid to interfaces. Several interfaces are available to couple the two systems online, such as on-column, loop-type and PTV interfaces which permit the transfer of large volume samples into the gas chromatograph. Insertion of a short trapping column between HPLC and GC also enables direct coupling from reversed-phase systems.

Fully Automated Dedicated Systems

The production of chemical compounds at the laboratory or plant scale requires fully automated and reliable separation techniques. This is true from discovery, through process control, to the final stage of purification and quality control of a new compound or drug. Numerous complete solutions exist today,

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such as online systems available in biotechnology, which involve automated process monitoring for the determination of small organic molecules, and interactive controls of the physicochemical and biochemical growth conditions in culture media. In many fields, including combinatorial chemistry, preparative separations are certainly widely used and require the most complete online solutions.

Preparative Chromatography

The objective of preparative chromatography is to obtain sufficient quantities of compounds at the required purity, with the highest throughput and at the lowest price. Material to be purified can be simple or more complex, such as fermentation broths and natural products. In such instances, fully automated preparative HPLC on a laboratory scale (milligrams to grams) is one of the best separation techniques for this objective, and also because of its flexibility to accommodate frequent application changes and variable quantity ranges.

The requirements of a preparative HPLC system differ according to the quantity of product to be collected, and the nature and number of samples to be purified. However it should provide reliable and complete automation for unattended operations with error-handling methods, and total traceability of all events, including the collected fractions. Fraction collectors are key instruments in preparative HPLC, and should be selected according to their collection facilities and fractionation software. Devices exist that can perform peak collection with automatic tracking of the baseline drift. With such instruments, collection is initiated if the detector signal is above user-set parameters in relation to threshold level and peak width. The graphic sample tracking which is feasible with some recent centralized software is also an important feature in preparative chromatography. Indeed, it can provide useful information to the user on sample identification, collection positions and associated chromatograms.

Since the introduction of independent programming of mobile-phase pressure, composition, and flow rate, SFC with packed columns has received considerable interest for several reasons, including its suitability and exceptional performances in preparative works. Consequently, this technique may become a serious competitor to preparative HPLC in the near future. **Figure 7** shows a completely automated system for analytical and preparative SFC. A versatile dual-valve autosampler of large capacity is used to perform both injection and collection. A solvent-less injection mode and a column-switching procedure are realized by two extra six-port valves. Such a system performs analytical development and switches easily and quickly to preparative chromatography for linear scale-up, and back to analytical for the purity check of the collected fractions. All these steps are automatically monitored by centralized software.

Analysis and Purification in Combinatorial Chemistry

Combinatorial chemistry appeared a few years ago in the pharmaceutical and biotechnological industries as

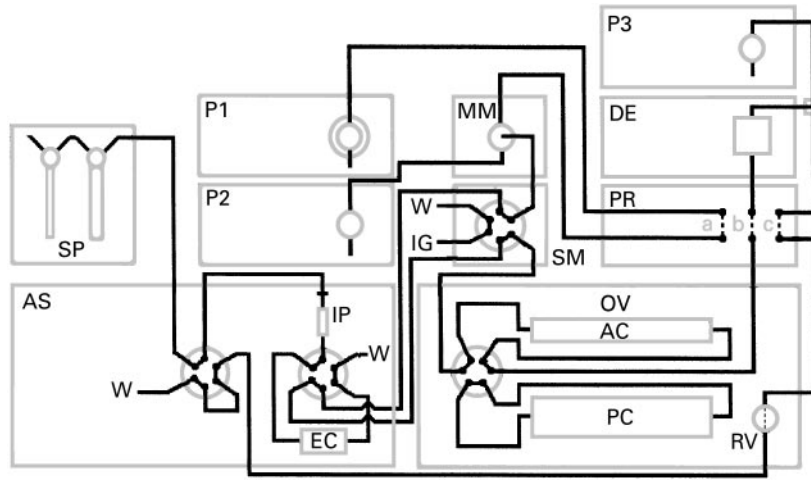

Figure 7 Automated analytical/preparative supercritical fluid chromatography on packed columns, with a solvent-less injection mode, based on Series SF3™ (Gilson) technology. AC, Analytical column; AS, dual-valve autosampler; DE, detector; EC, enrichment cartridge; IG, inert gas; IP, injection port; MM, mixing module; OV, oven; PC, preparative column; PR, pressure regulator (a, upstream pressure and pulse damper; b, heat exchanger; c, downstream pressure regulator); P1, liquid carbon dioxide pump; P2, modifier pump; P3, collection solvent pump; RV, pressure relief valve; SM, switching valve module; SP, dual-syringe pump; W, waste.

a novel approach to finding new drugs, and has undergone outstanding development since then. It is defined by a variety of automated high throughput synthesis techniques, to produce large numbers of small organic molecules, for screening these compounds against several biological targets for drug discovery. Combinatorial chemistry includes three basic steps – synthesis, candidate screening and library product purification – but chromatography is principally involved in the last two.

In drug candidate screening, library product verification can be performed with various analytical techniques. Among these, gradient reversed-phase HPLC with UV detection is widely used. Such a system, fitted with an autoinjector which accommodates microtitre plates, provides a robust and highly automated approach. Fast HPLC methods (generally of less than 5 min) generate up to 300 analyses per day per system and are required to produce libraries of products with widely differing polarity. These generic methods use short C_{18} columns with sharp slope gradients from acidified water to acidified organic solvents, high flow rates and UV detection set at 220 or 254 nm. Automated HPLC-MS, HPLC-DAD-MS and HPLC-MS-MS have also become important routine tools for the combinatorial chemist, because they provide structural information to identify the compounds which have been synthesized.

Automated purification to an extremely high level with sample quantities of up to 100 mg can be achieved with a preparative HPLC system equipped with an autoinjector and a fraction collector. The technique uses the same packing material and mobile phases as those used in analytical separation. Fast and easy clean-up can also be performed using SPE cartridges or microplates. Samples may therefore be processed in parallel with an automated device for optimum throughput. And last but not least, preparative SFC, although with limited applications so far, also provides a high productivity approach to purification, and in the near future should acquire a much bigger place in combinatorial chemistry.

Future Trends

Automation in chromatography has been a major preoccupation in recent years, mostly for pharmaceutical and clinical laboratories, and this will certainly be more intensive in the future, with concomitant developments in other fields like chemical, agrochemical, food or environmental analysis. The main goal is to provide more analyses giving the maximum and the most accurate information in the shortest time, at the lowest cost. This is the current situation in pharmaceutical and biomedical research for high throughput screening in combinatorial chemistry, genomics or proteomics. Future trends in automation will surely follow the evolution of instruments, including mixed-mode techniques like capillary electrochromatography, more efficient interfaces, and more versatile and user-friendly software. They will also follow the progress in chromatographic media like restricted-access sorbents or advanced stationary phases with sub 3 μm particles for LC, and new materials for sample preparation such as 96-well SPE extraction plates.

Miniaturization in chromatography, including automated micro- and nano-techniques, is certainly an important step towards this goal, and a good example is given by the fast separation of complex mixtures with miniature gas chromatographs, which can now be performed in the field. Moreover, in addition to allowing the analysis of very limited volume samples with good sensitivity, and to reduce mobile-phase consumption, miniaturization tends to reduce work space, robot displacements and liquid transfers and consequently overall analysis time. The evidence of the release on the market of 384-well SPE extraction plates will obviously confirm this trend. Down-sizing even further, the use of 1536-well microtitre plates automated by sample processors equipped with distribution heads and chip format technology will certainly be expanded in the very near future. This goal involves refinement of the technique to carry out continuous separations with sub-second time resolution and attomole detection limits.

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See also: **II/Chromatography:** Liquid Chromatography - Gas Chromatography. **Chromatography: Gas:** Historical Development; Detectors: Mass Spectrometry. **Chromatography: Liquid:** Detectors: Mass Spectrometry; Instrumentation; Large-Scale Liquid Chromatography. Multidimentional Chromatography. **Electrophoresis:** Capillary Electrophoresis. **Extraction:** Solid-Phase extraction; Solid-Phase Microextraction; Supercritical Fluid Extraction.

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Convective Transport in Chromatographic Media

A. E. Rodrigues, LSRE Faculty of Engineering, University of Porto, Porto, Portugal

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New liquid chromatographic media have been developed in the last decade containing large pores for convective mass transport and smaller diffusive pores to provide adsorption capacity. These permeable packings are at the heart of perfusion chromatography, patented in 1991, leading to better column efficiency and speed of separation with applications in the rapid analysis of biological macromolecules and preparative scale purification of proteins. This improved column efficiency is based on the concept of augmented diffusivity by convection, available from parallel development in the chemical reaction engineering area.

The analysis of column performance requires an extended van Deemter equation for the HETP (height equivalent to a theoretical plate) as a function of the superficial velocity. A methodology for obtaining basic data for design of perfusive chromatography is suggested involving elution chromatography of proteins in nonretained and frontal chromatography experiments.

Permeable Chromatographic Media

Packing materials in bead form can be grouped into four classes: homogeneous cross-linked polysaccharides (e.g. agarose), macroporous polymers based on synthetic polymers (e.g. POROS particles used in perfusive chromatography with large pores of 600–800 nm and diffusive pores of 50–100 nm), tentacular adsorbents allowing faster interaction between proteins to be separated and functional groups and materials based on the concept of 'soft gel in a rigid shell' combining the good capacity of soft gels with the rigidity of composite materials.

In conventional packings, solutes are carried to sites on the particle surface by bulk convective flow of the mobile phase through the column and then diffuse to binding surfaces inside the particle. The intraparticle diffusion process can be quite slow for large molecules (proteins, peptides). In order to maximize capacity and resolution, interaction with as many sites as possible is required; however, this will be more difficult at high flow rates and therefore trade-offs between speed, resolution and capacity are needed. In permeable, flow-through particles used in perfusive chromatography the flow rate can be increased one order of magnitude compared with conventional packings because of the short diffusion path length inside the microspheres.

New materials aim to achieve higher sorption capacity and better sorption kinetics. Some developments consider new geometries: fibres, membranes or discs (cellulose, polymethacrylate) or continuous beds (rods, monoliths). In continuous rods of acrylamide-acrylate polymers, flow pores are of 3–4 μm diameter and in methacrylate-styrene polymers large pores of 0.5–2 μm and even 20 μm are obtained with microspheres of less than 0.5 μm. Similar developments on continuous silica rods are taking place. In continuous bed technology, channels are of 3–5 μm with microspheres of 0.5–1 μm, whilst in bead form, packings are typically of 3–50 μm with pore size of 0.1 μm. Also superagarose beads of 300–500 μm have been prepared with superpores of 30 μm as well as 3 mm thick membranes. **Table 1** reports examples of convective chromatographic media.

The Concept of Augmented Diffusivity by Convection

The design of chromatographic media aims to eliminate or reduce the mass transfer resistance inside particles by coating a nonporous support with active species, decreasing the particle size or increasing par-